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ORIGINAL ARTICLE

Polar components of *Phaleria macrocarpa* Fruit Exert Antihypertensive and Vasorelaxant Effects by Inhibiting Arterial Tone and Extracellular Calcium Influx

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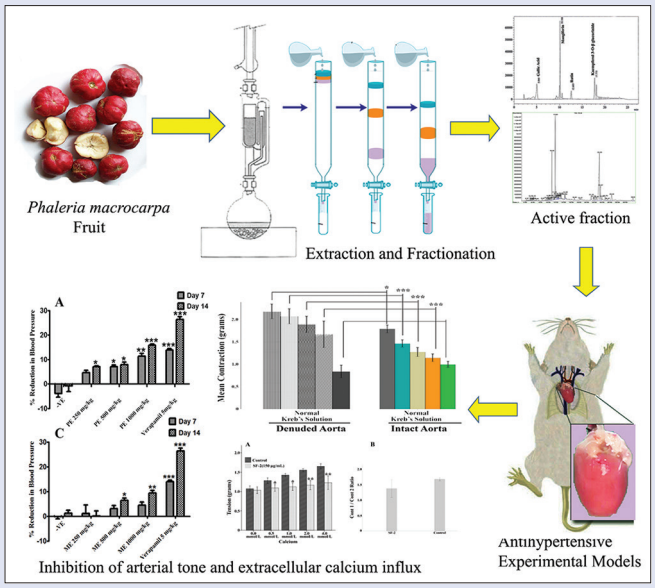
ABSTRACT

Background: *Phaleria macrocarpa* (PM) has been used conventionally to cure hypertension. **Objective:** The objective of the study is to determine the active fraction and its chemical composition responsible for antihypertensive activity of PM fruit using a bioactivity-guided investigation on different extracts of the fruit. **Materials and Methods:** Among the extracts of PM, water extract (WE) showed prominent effect in screening test by inhibiting noninvasive blood pressure (BP) in spontaneously hypertensive rats (SHRs). WE was investigated further using hypertensive and normotensive experimental models. **Results:** WE caused dose-dependent hypotensive effect in normotensive rats with adrenergic and cholinergic effects by inhibiting the elevated levels of norepinephrine hydrochloride and acetylcholine hydrochloride induced mean arterial pressure (MAP) and heart rate (HR). In addition, WE enhanced the activity of nonselective β-agonist isoprenaline on MAP and inhibited the increased HR. Similarly, WE demonstrated significant inhibition on pulse wave velocity (PWV), MAP, and HR in SHRs. Fractions of WE were tested for vasorelaxation effect on rat aortic explant. Among the fractions, water fraction-4 (PF-4) showed pronounced effects. Column chromatography of PF-4 yielded two subfractions; among them, sub-fraction-2 (SF-2) displayed significant vasorelaxation effect in endothelium-denuded and endothelium-intact aortas. Further, SF-2 revealed significant inhibition in calcium influx and mobilization from intracellular stores. Gas chromatography-mass spectroscopy analysis of SF-2 revealed abundance of kaempferol 3-O-β glucuronide, mangiferin, gallic acid, and rutin. **Conclusions:** This study demonstrates that polar phytochemical fraction of PM fruit has a promising potential of reducing PWV, BP, and HR. This antihypertensive effect is probably due to the inhibition of arterial tone and extracellular calcium influx.

Key words: Arterial stiffness, isolated aorta, *Phaleria macrocarpa*, phytochemistry, spontaneously hypertensive rats, thoracic cannulation

SUMMARY

- *Phaleria macrocarpa* (PM) has been used conventionally to cure hypertension. In this study, a bioactive-guided extraction and fractionation was conducted on PM fruit using antihypertensive assay. Among the extracts of PM, water extract (WE) showed prominent effect in screening test. Therefore, further, the WE was investigated using hypertensive and normotensive experimental models. WE caused dose-dependent hypotensive effect in normotensive rats with adrenergic and cholinergic effects by inhibiting the elevated levels of norepinephrine hydrochloride and acetylcholine hydrochloride induced mean arterial pressure (MAP) and heart rate (HR). In addition, WE enhanced the activity of nonselective β-agonist isoprenaline on MAP and inhibited the increased HR. Similarly, WE demonstrated significant inhibition on pulse wave velocity (PWV), MAP, and HR in SHRs. Fractions of WE were tested for vasorelaxation effect on rat aortic explant. Among the fractions, platelet factor 4 (PF-4) showed pronounced effects. Column chromatography of PF-4 yielded five subfractions; among them, SF-2 displayed significant vasorelaxation effect in endothelium-denuded and endothelium-intact aortas. Further, SF-2 revealed significant inhibition in calcium influx and mobilization from intracellular stores. Gas chromatography-mass spectroscopy analysis of SF-2 revealed abundance of kaempferol 3-O-β glucuronide, mangiferin, gallic acid, and rutin. Altogether, it can be concluded that the polar components of PM fruit have a promising potential of reducing PWV, blood pressure, and HR. This antihypertensive effect is probably due to the inhibition of arterial tone and extracellular calcium influx.



Abbreviations used: WE: Water extract; SHRs: Spontaneously hypertensive rats; NE-HCl: Norepinephrine hydrochloride; MAP: Mean arterial pressure; HR: Heart rate.

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INTRODUCTION

The extracts of *Phaleria macrocarpa* (PM) (Scheff.) Boerl. have been reported for many pharmacological properties such as anti-inflammatory,[1-3] antimicrobial,[3,4] antioxidant,[4-6] cytoprotective,[7] and antidiabetic effects. Earlier studies report the traditional use of the dried fruit and seed shells of PM for the management of hypertension in Indonesian and Malaysian folk medicine.[8-11] The three major constituents isolated from the PM fruit extracts include mangiferin, icariside C3, and gallic acid.[12] The well-accepted antihyperlipidemic potential of the herb could be attributed to gallic acid that has made the extensive use of PM extracts to treat vascular disorders.[13,14] It is well established that gallic acid upregulates the low-density lipoprotein receptors and pro-protein convertase subtilisin/kexin type 9 expression in cells that account for the reduction in cholesterol level.[14]

It is reported that icariside C3, which is a mild vasodilator, has been isolated from the chloroform extract of PM fruits that inhibited norepinephrine-induced contraction in isolated rat aorta.[12] More recently, the hypotensive and hypoglycemic effects of the methanol extract of PM fruits are reported in spontaneously hypertensive rats (SHRs).[15] However, PM extracts have never been investigated in a bioactivity-guided manner to identify the active constituents responsible for vasorelaxant and hypotensive efficacies. Moreover, the antihypertensive effect of PM extracts in hypertensive rats has never been compared with that in the normotensive rats, and the underlying mechanism of its vasorelaxant effect has not yet established. Rats are the suitable experimental model for human hypertension studies because the pathogenesis of hypertension in rats and humans are mostly similar in terms of arterial pressure development and its response to various stimuli. The current research was therefore designed to investigate the bioactivity-guided extraction and fractionation of PM fruit to characterize the vasorelaxant constituents present in the fruit and to elucidate the underlying mechanism of antihypertensive efficacy in hypertensive and normotensive rats.

MATERIALS AND METHODS

Chemicals and instruments

Norepinephrine hydrochloride (NE-HCl), acetylcholine hydrochloride (Ach-HCl), verapamil hydrochloride, isoprenaline hydrochloride, atropine sulfate, N-nitro-L-arginine methyl ester hydrochloride (L-NAME), indomethacin, and propranolol hydrochloride were purchased from Sigma Aldrich (Seelze, Germany). Data acquisition system (PowerLab) was purchased from ADInstruments (Bella Vista, Australia). Rotavapor Model R-210/215 was purchased from Buchi (Flawil, Switzerland). Petroleum ether, chloroform, and methanol were purchased from Fisher Scientific, UK. The organic solvents used were of high-performance liquid chromatography (HPLC) grade.

Plant material

PM fruits were collected from a commercial supplier in Pulau Pinang, Malaysia. The herb was identified, and a voucher specimen was submitted in the herbarium, School of Biological Sciences, Universiti Sains Malaysia (voucher number 11259).

Preparation of the crude extracts

The scheme used for the extraction of PM fruits is shown in supplementary material [Figure S1]. Approximately 3 kg of powdered dry fruit was sequentially extracted in petroleum ether, chloroform, and methanol using a Soxhlet apparatus. The residue was macerated with distilled water at 45°C overnight. The resultant four extracts, i.e., petroleum ether extract (PE, 134.4 g), chloroform extract (CE 56.34 g), methanol extract (ME, 239.8 g), and water extract (WE, 342.8 g), were filtered, concentrated in a rotary evaporator, freeze dried, and then stored in a desiccator until further use.

Fractionation of crude extracts

PE was subjected to dry-column flash chromatography as described earlier.[16] The column was first eluted with 2 × 100 mL of n-hexane, followed sequentially with 2 × 100 mL of four solvents: n-hexane: chloroform (3:1); n-hexane: chloroform (1:1); n-hexane: chloroform (1:3), and chloroform. Fixed volume elutions were collected and examined with thin-layer chromatography (TLC) using n-hexane: ethyl acetate (9:1) solvent system, and the eluents with similar TLC profiles were pooled together yielding two subfractions designated as platelet factor (PF)-1 23.4 g (26%) and PF-2 36.17 g (40.18%), respectively.

WE (250 g) was subjected to partition liquid fractionation by following the method advised earlier with some modifications.[17] The resultant chloroform fraction (WF-1, 18.4 g), ethyl acetate fraction (WF-2, 42.7 g), *n*-butanol fraction (WF-3, 38.9 g), and the residual water fraction (WF-4, 79.6 g) were concentrated, freeze dried, and stored in the desiccator until further use.

Sub-fractionation

Further fractionation of WF-4 was carried out by column chromatography as described earlier with minor modifications.[18] The elutions obtained (chloroform, chloroform: methanol, methanol, and methanol: water) were subjected to TLC, and the elutions with similar Rf values on TLC were pooled up together to obtain two subfractions SF-1 (278.4 mg) and SF-2 (760 mg).

Phytochemical analysis

Gas chromatography/mass spectroscopy analysis of petroleum ether fractions

Analysis of PF-1 and PF-2 was carried out by gas chromatography-mass spectroscopy (GC-MS) using Agilent 6890 gas chromatography instrument coupled to Agilent 5973 mass spectrometer and Agilent ChemStation software (Agilent Technologies, Palo Alto, CA, USA). The constituents of the fractions were separated on HP-5MS capillary column (30 m × 0.25 mm ID × 0.25 μm, film thickness). The oven temperature was held at 70°C for 2 min followed by an increase up to 285°C (at a rate of 20°C/min) that was maintained for 20 min. Helium (1.2 mL/min flow rate) was used as a carrier gas. The injector and detector temperatures were set to be 250°C and 285°C, respectively. Mass detection was performed at ion mass/charge ratio 35–550 m/z where the scan mode and the ionization energy were 70eV.

High-performance liquid chromatography analysis of SF-2

Four standards, i.e., gallic acid, mangiferin, rutin, and kaempferol 3-O-β-D-glucuronide, were used to standardize SF-2 by HPLC. The separation was carried out using a reversed phase C-18 Agilent Eclipse Plus (Agilent, USA) column (250 × 4.6 mm ID, 5 μm). The flow rate was set at 1.2 mL/min and detection wavelength was 280 nm. The sample of 25 μL was injected onto the column.

Different composition of mobile phase was studied to achieve good resolution and short elution time. The mobile phase was filtered through Whatman filter 0.45 μm (nylon membrane filters 47 mm) and degassed before use. Reversed-phase HPLC technique was used to measure constituents of SF-2 quantitatively based on the method described earlier.[19]

Pharmacological procedures

Animals

SHRs and Wistar Kyoto (WKY) rats weighing 200–250 kg were obtained from the Animal Research and Service Centre, Universiti Sains Malaysia, after approval from the Animal Ethics Committee of Universiti Sains Malaysia. The animals were kept in an animal transit room with free access to water and food *ad libitum* in a 12-h light and dark cycle.

Acute toxicity study in rat

Healthy adult female Sprague–Dawley rats (200–250 g) were used in the acute toxicity study according to the fixed dose procedures by following the OECD guidelines 420.[20] After an overnight fast, one animal from four groups containing five rats in each was administered a single dose (2000 mg/kg) of PE, CE, ME, and WE, respectively. After this single-dose administration, signs of possible toxicity were observed for the first 6 h and then daily for 14 days. The same dose of the extract was given to additional four rats from each group if the first animal survived after 48 h of treatment. The animals were kept under observation for any signs of toxicity or changes in behavioral patterns or physical changes, and their weight was measured for comparison on day 0, 7, and 14.

Screening of antihypertensive effect of Phaleria macrocarpa extracts

The preliminary antihypertensive assessment of PM crude extracts was screening by measuring noninvasive blood pressure (NIBP) in six groups of rats as reported earlier.[21] The vehicle (1% Tween 80) and verapamil (5 mg/kg) were administered to Group A rats (10 mL/kg) and Group F, respectively, whereas Groups B, C, D, and E rats were treated with PE, CE, ME, and WE, respectively, each in three doses, i.e., 250, 500, and 1000 mg/kg, thus dividing each group of rats from Group B to Group E into three subgroups with six animals in each.[22] The blood pressure (BP) of the rats was recorded by data acquisition system (PowerLab, ADInstruments) on the day 0, 7, and 14 of the treatment, and the percent reduction in BP was recorded for each group using the following formula:

$$\% \text{ reduction in BP} = \left[1 - \left(\frac{\text{BP on day B}}{\text{BP on day A}}\right)\right] \times 100$$

where day B represents day 7th or day 14th and day A shows day 0 (starting day).

Effect on pulse wave velocity in spontaneously hypertensive rats

Rats divided in five groups (Groups A, B, C, D, and E were used to evaluate the effect of WE of PM on pulse wave velocity [PWV], mean arterial pressure [MAP], and heart rate [HR] in SHRs). Groups A and B were administered with water (10 mL/kg) and verapamil (5 mg/kg) as control and reference, respectively, for 3 weeks. Groups C, D, and E were administered with 250, 500, and 1000 mg/kg of WE, respectively, for 3 weeks.

All the rats were cannulated on the 21st day by thoracic cannulation involving right carotid artery and femoral artery following the procedures advised earlier.[23] The length between the two transducers connected to the two arteries was measured with the help of thread and scale designated as the wave propagation distance "*L*." The wave propagation time "*t*" was estimated using diastolic phase center technique. The time at which the pulse wave crossed 1 mmHg above the minimum pressure point in both the upslope and downslope of the wave was averaged, and this average time value from transducer 1 wave was subtracted from the averaged time from transducer 2 wave. This process was repeated on nine cycles, and the average of the nine values was taken as the wave propagation time. PWV was calculated by using the following formula:

$$\text{PWV} = \frac{\text{L}}{\text{t}}$$

Adrenergic and cholinergic effects of Phaleria macrocarpa water extract

The WKY rats divided in eight groups (six rats in each group) were cannulated by thoracic cannulation involving left jugular vein and right carotid artery after being anesthetized with ketamine (85 mg/kg) and xylazine (5 mg/kg) following the procedures reported earlier with minor modifications.[24] MAP and HR were recorded after stabilization for 30–40 min.

In Group A rats, changes in MAP and HR were recorded with increasing doses of WE at the doses of 3, 6, 12, 25, 50, and 100 mg/kg administered as intravenous bolus injections through jugular vein in a volume of 0.1 mL followed by 0.1 mL flush of saline to ensure complete delivery of the dosage.[25] In Group B rats, the values of MAP and HR were recorded after administration of NE-HCl at the doses of 0.15, 0.30, and 0.60 μg/kg. The similar increase in MAP and HR by the above-mentioned doses of NE-HCl was recorded following a single dose of 1 mg/kg phentolamine (Group B), 50 mg/kg WE (Group C), and 1 mg/kg verapamil (Group D) as advised earlier.[26] The values of MAP and HR were recorded after administration of Ach-HCl at the doses of 0.5, 1.0, and 2.0 μg/kg. The similar effect of Ach-HCl in the above-mentioned doses on MAP and HR was recorded following a single dose of 1 mg/kg atropine (Group E) and 50 mg/kg WE (Group F).

The values of MAP and HR were recorded after administration of isoprenaline at the doses of 0.05, 0.1, and 0.2 μg/kg. The similar effect of isoprenaline in the above-mentioned doses on MAP and HR was recorded following a single dose of 2 mg/kg propranolol (Group G) and 50 mg/kg WE (Group H).

Effect of Phaleria macrocarpa extract on isolated aortic rings precontracted with norepinephrine

The rats were euthanized in carbon dioxide (CO_2) chamber, and the aorta was excised immediately. The isolated aorta cut into 3 mm wide rings were suspended in tissue organ bath containing Krebs-Henseleit solution aerated with 95% O_2 and 5% CO_2 and maintained at 37°C under a resting tension of 1 g. Changes in tension were recorded by the connected PowerLab of Quad Bridge Amplifier (ADInstruments) ML 224. After achieving stabilization (in almost 1.5 h), contractions were recorded by instilling 100 μL of 10^{-6} M NE-HCl. Integrity of intact endothelium was confirmed by observing 70% relaxation after instilling 100 μL of 10^{-6} M Ach-HCl.[27]

The crude extracts/fractions/subfractions of PM were added cumulatively in the concentrations of 0.25, 0.5, 1.0, and 2.0 mg/mL to the organ bath after attaining plateau.[27] The tension was measured, and the cumulative concentration–response curves were recorded using force–displacement transducer coupled with data acquisition system (PowerLab, ADInstruments). All the experiments were performed in six replicates. The results were presented as percent relaxation that was calculated by the following formula:

$$\% \text{ Relaxation} = \left[1 - \left(\frac{\text{Contraction in treated rings}}{\text{Contraction in untreated rings}}\right)\right] \times 100$$

Assessment of vasorelaxant mechanism of SF-2

The possible involvement of vascular endothelium in the vasorelaxant effect of SF-2 was evaluated in the aortic tissue with intact endothelium as well as with denuded endothelial lining. Three set of experiments were performed to evaluate the involvement of prostacyclin (PG) and nitric oxide (NO) pathway.

The aortic tissue with intact endothelium was incubated with indomethacin 10^{-5} M for 15 min in the tissue organ bath before contracting

the aortic rings with NE-HCl (10^{-6} M) to ascertain the involvement of PGI_2 pathway.[27-29] Then, SF-2 was added at the concentrations of 0.25, 0.5, 1, and 2 mg/mL cumulatively to obtain the dose–response curve.

The aortic tissue was incubated with L-NAME 10^{-4} M for 15 min before the rings were contracted with NE-HCl (10^{-6} M).[28,30] The SF-2 was then added at the concentrations of 0.25, 0.5, 1, and 2 mg/mL cumulatively and dose–response curves were obtained.

The endothelium of aortic ring was denuded by gently rubbing the intimal space of endothelial lining with the teeth of forceps.[31] It was confirmed by the failure of relaxation by Ach-HCl (10^{-6} M) added in the tissue organ bath on the rings precontracted with NE-HCl (10^{-6} M). Dose–response curves were obtained by instilling SF-2 at the concentrations of 0.25, 0.5, 1, and 2 mg/mL cumulatively in the tissue organ bath on the precontracted rings (with 10^{-6} M NE-HCl). The % relaxation induced by SF-2 in aortic rings was calculated using the following formula:

$$\% \text{ Relaxation} = \left[1 - \left(\frac{\text{Contraction in treated rings}}{\text{Contraction in untreated rings}}\right)\right] \times 100$$

The percentage relaxation induced by SF-2 in intact aortic tissue preincubated with indomethacin and L-NAME was compared with % relaxation recorded in the tissue with denuded endothelium to conclude the involvement of PGI_2 or NO in the vasorelaxant effect of SF-2.

Effect of SF-2 on calcium influx

Two different sets of experiments were performed on denuded isolated aortic tissue.

Effect of SF-2 on extracellular calcium influx

To verify whether the relaxation in the aortic rings involved calcium influx, the denuded aortic tissue was washed 4–5 times in calcium-free Kreb's solution and could stabilize. NE-HCl (10^{-6} M) was added before incubation with SF-2 to produce a steady contraction.[27] The tissue was then incubated with 2 mg/mL SF-2 for 10 min before the addition of cumulative dose of calcium (0.01–3 mM) to obtain the dose–response curve.

Effect of SF-2 on intracellular calcium release

To assess the effect of SF-2 on the mobilization of calcium from intracellular stores, the denuded aorta were incubated in calcium-free Kreb's solution containing 118 mM/L sodium chloride, 4.7 mM/L potassium chloride, 1.2 mM/L magnesium sulfate, 1.2 mM/L potassium biphosphate, 10 mM/L glucose, 25 mM/L sodium bicarbonate, and 1.5 mM/L of ethylene glycol tetraacetic acid (EGTA) for 15 min. NE-HCl (10^{-6} M) was instilled thereafter to produce a steady-state contraction (con1). The aorta was washed with normal Kreb's solution and incubated for 40 min. Again, the aorta was incubated with calcium-free medium for 15 min, and NE-HCl (10^{-6} M) was instilled to record the second contractions (con2) after preincubating the baths with or without SF-2 for 10 min. The ratio of con2 over con1 was calculated.

Statistical analysis

Data obtained from isolated rat thoracic aorta experiments were expressed as the mean ± standard error of the means. Statistical difference between the treatments and the control was evaluated by one-way analysis of variance followed by Tukey's HSD *post hoc* test. Differences were considered significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

RESULTS

The four crude extracts did not show any treatment-related sign of toxicity during 2 weeks of observation after the administration of a single oral dose and the extracts were found to be safe at 2 g/kg. Figure 1 shows % inhibition of BP in SHR by the four crude extracts. Although PE [Figure 1a] and WE [Figure 1d] both exhibited significant antihypertensive effect in SHRs on day 7 and 14 ($P < 0.05$), the activity of WE was comparable with that of verapamil ($P > 0.05$). CE did not

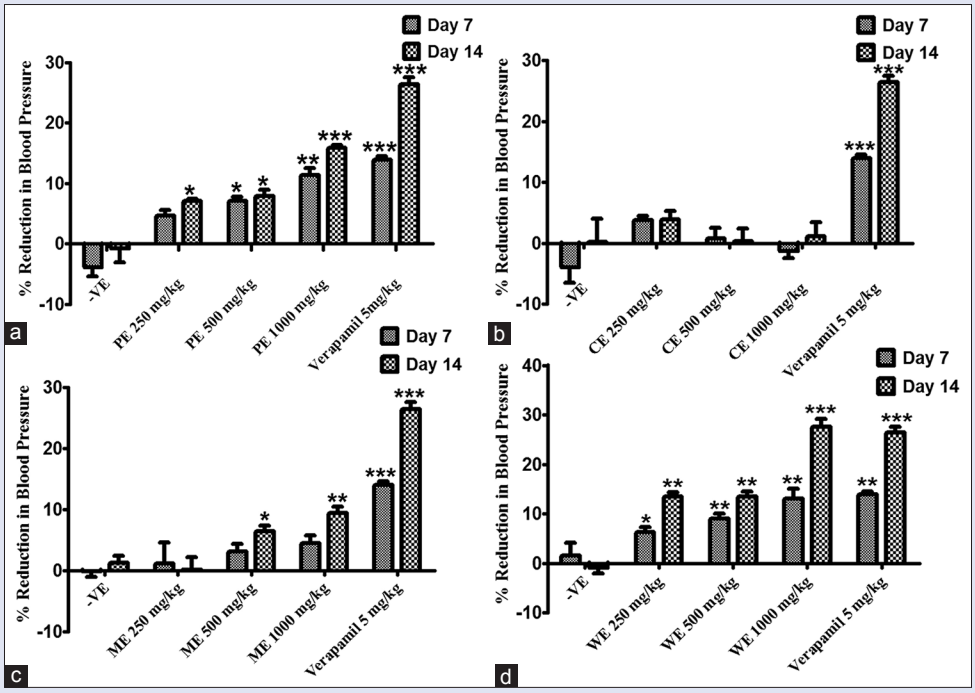

Figure 1: Antihypertensive effect of *Phaleria macrocarpa* extracts in spontaneously hypertensive rats. (a-d) The percent inhibition of blood pressure on the 7th and 14th day of treatment with petroleum ether, chloroform, methanol, and water extract, respectively. *, **, and ***indicate significant differences between control and treatment group at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively

show considerable activity even on the 14th day of treatment [Figure 1b], whereas ME caused a mild fall in pressure on the 14th day at higher doses of 500 mg/kg and above [Figure 1c].

Further, the WE showed significant inhibition in the elevated levels of NE-HCl-induced MAP and HR in WKY rats [Figure 2a and b; $P < 0.05$] in the similar way as that of the phentolamine and verapamil [Figures S2 and S3]. The extract (WE) enhanced the activity of the nonselective β-agonist isoprenaline on MAP and inhibited the increased HR at its higher doses [Figures S4 and S5]. In addition, the extract caused significant suppression of Ach-HCl effect on MAP and HR when given with Ach-HCl as presented in Figure S5. Figure 3a and b represents the effect of WE on HR and MAP, respectively, in SHRs after 3 weeks oral administration. Figure 4 shows the effect of WE on the PWV. The results showed that treatment with WE up to the dose of 500 mg/kg did not produce a significant decrease in HR, MAP, and PWV. However, a significant fall in HR ($P < 0.05$), MAP ($P < 0.01$), and PWV ($P < 0.01$) was observed at the higher dose of 1000 mg/kg.

The vasorelaxant effect of the four crude PM extracts was evaluated in the aortic explant of rat which was precontracted with NE-HCl and the results are presented in Figure 5a. In terms of percent relaxation, CE failed to relax the rat aortic tissue, whereas a moderate relaxation effect was observed with ME ($P < 0.05$). PE and WE produced profound relaxation activity ($P < 0.001$), in which WE being the most potent extract among all the four crude extracts tested. The PF-2 fraction of PE showed mild effect at the highest dose of 2.0 mg/mL [Figure 5b] which was statistically ($P < 0.05$) significant; however, PF-1 fraction did not produce significant relaxation effect. Then, WE was subjected to fractionation, and its fraction WF-4 exhibited the most significant activity among all the four fractions [Figure 5c; $P < 0.05$]. Therefore, the fraction WF-4 was further fractionated into SF-1 and SF-2. The subfraction SF-2 displayed the most significant activity in all the tested concentrations ranging from 0.25 to 2.0 mg/mL [Figure 5d; $P < 0.05$].

Figure 6a represents NE-HCl-induced contraction (presented in grams) in the aortic tissue with denuded as well as intact endothelium. The effect of the four doses of SF-2, i.e., 0.25, 0.5, 1, and 2 mg/mL, on the NE-HCl-induced contraction was statistically compared between the groups. Figure 6a clearly shows that SF-2 exhibited significant relaxation in the denuded as well as the intact aorta; however, the effect was comparatively weak in the denuded aorta and the lower concentrations caused less vasorelaxation compared to that in the aorta with intact endothelium. Incubation with L-NAME showed a pattern of activity that was very similar to that in the denuded aorta [Figure 6b]. The effect of SF-2 in the aortic tissue preincubated with L-NAME was significantly less as compared to that recorded in the absence of L-NAME. However, this reduction in activity was noted only at SF-2 concentrations ranging from 0.25 to 1.0 mg/mL. At the concentration of 2.0 mg/mL, the vasorelaxant effect of SF-2 was statistically more significant ($P < 0.05$) in the presence of L-NAME as compared to that in its absence. Incubation of intact aortic rings with indomethacin significantly reduced the activity of SF-2 in concentrations ranging from 0.25 to 0.5 mg/mL [Figure 6c, $P < 0.01$].

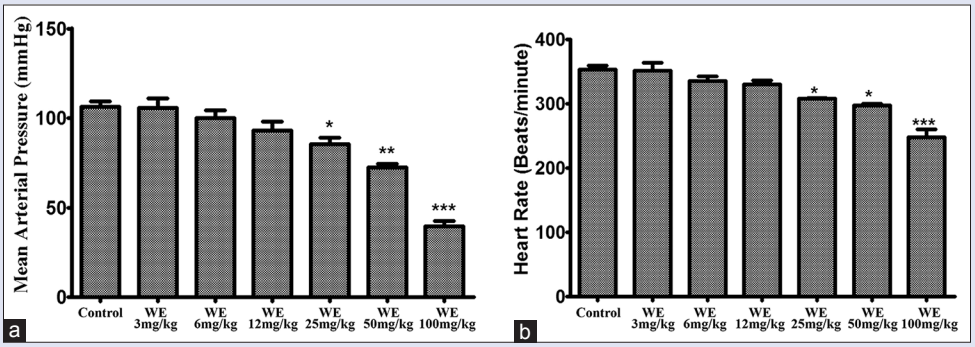

Figure 2: The effect of intravenous administration of water extract of *Phaleria macrocarpa* on the mean arterial pressure (a) and heart rate (b) of anesthetized Wistar Kyoto Rats. The results are presented as mean ± SEM. *, **, and ***indicate significant differences between control and treatment group at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. SEM: Standard error of the mean

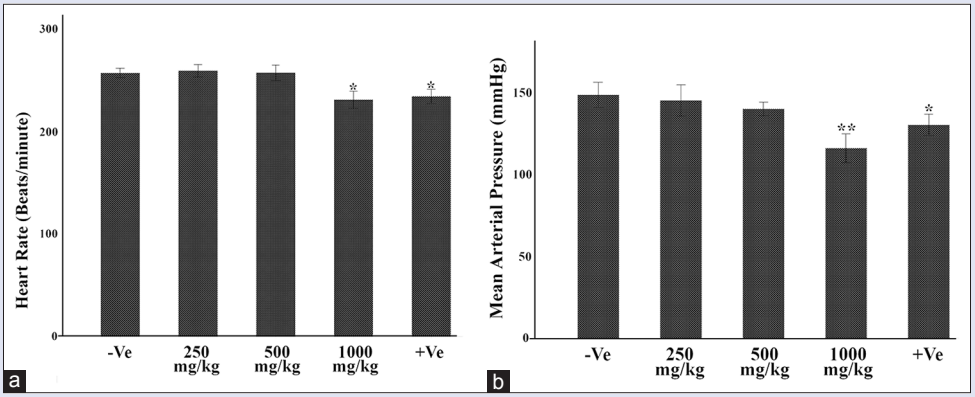

Figure 3: The effect of 3 weeks dosing of *Phaleria macrocarpa* water extract on the heart rate (a) and mean arterial pressure (b) of spontaneously hypertensive rats. –Ve shows the control group rats whereas + Ve represents reference group treated with verapamil. Results are presented as mean ± SEM. * and **represent the statistical difference between the control and treatment groups at the significance of $P < 0.05$ and $P < 0.01$, respectively. SEM: Standard error of the mean

However, at higher concentrations, SF-2 did not exhibit significant relaxation.

Figure 7a represents the contraction recorded in the aortic tissue pretreated with SF-2 upon the addition of cumulative concentrations of calcium. Addition of calcium gradually increased the contraction in aortic smooth muscle in control as well as in the treatment group; however, this increase in contraction was lesser in SF-2-treated tissue as compared to the untreated group ($P < 0.05$). Figure 7b depicts the comparison between the effect of SF-2 incubation on the aortic rings precontracted with NE-HCl in both the presence and absence of EGTA. The results in the SF-2-treated group were quite as that of the control ($P > 0.05$).

Tables S1 and S2 depict the qualitative analysis of PF-1 and PF-2, respectively. The most dominant constituent of PF-2 was octadec-9-enoic acid that constituted 24.2% of the fraction. The GC-MS chromatograms of fraction PF-1 and PF-2 are provided as supplementary files [Figures S6 and S7].

The concentration of the four constituents, i.e., kaempferol 3-O-β glucuronide, mangiferin, gallic acid, and rutin, in 44 mg of SF-2 was found to be 1.73, 0.21, 0.04, and 0.04%, respectively. The peaks of the four constituents in SF-2 with their respective retention times on the HPLCs are presented in Figure S8a, whereas the reference peaks of the standards are presented in Figure S8b.

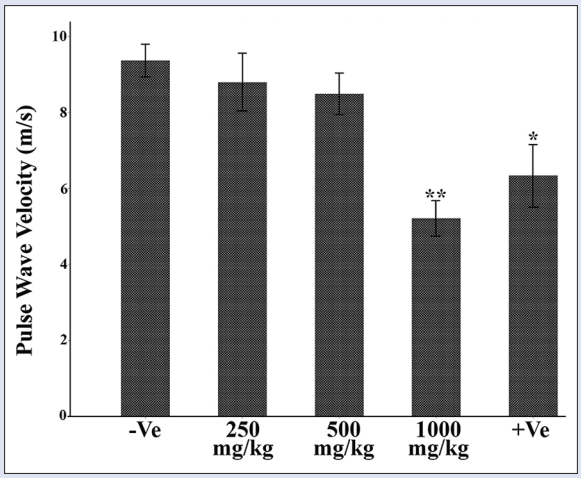

Figure 4: The effect of 3 weeks dosing of *Phaleria macrocarpa* water extract on the pulse wave velocity of spontaneously hypertensive rats. –Ve shows the control group rats whereas + Ve represents reference group treated with verapamil. Results are presented as mean ± SEM. * and **represent the statistical difference between the control and treatment groups at the significance of $P < 0.05$ and $P < 0.01$, respectively. SEM: Standard error of the mean

DISCUSSION

Hypertensive studies weak

The dried PM fruits were sequentially extracted using petroleum ether, chloroform, methanol, and water to obtain the phytoconstituents based on their differential polarities. The highly nonpolar and highly polar constituents resided in PE and WE, respectively, whereas the constituents with intermediate to high polarity were concentrated in CE and ME, respectively. The crude extracts were given to SHRs at the doses of 250, 500, and 1000 mg/kg/day for 14 days, and the BP of the rats was measured on day 0, 7, and 14 by tail-cuff method. Out of the four crude extracts, WE significantly reduced BP in rats on the 7th day of treatment at all the three given doses. The lowest dose of PE, i.e., 250 mg/kg/day, exhibited a significant antihypertensive effect only

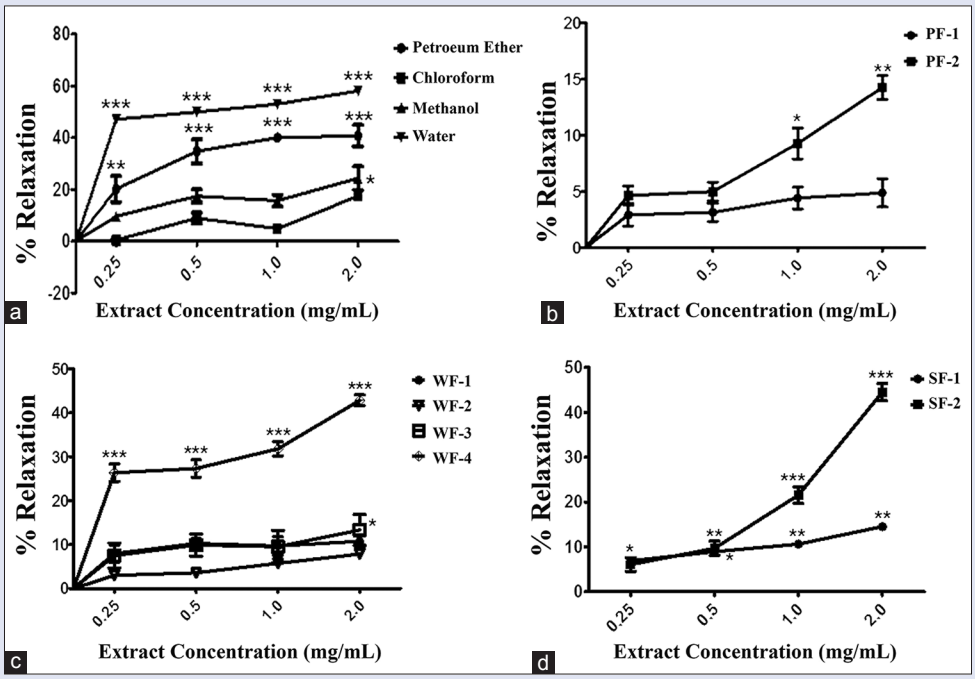

Figure 5: Inhibition of norepinephrine-induced contraction in isolated rat aorta by *Phaleria macrocarpa* extracts. (a) The percent relaxation in precontracted rat aorta treated with petroleum ether extract, chloroform extract, methanol extract, and water extract. (b) The percent relaxation recorded in aortic tissues that were treated with petroleum ether fractions PF-1 and PF-2 whereas (c) the same results for water extracts fractions WF-1, WF-2, WF-3, and WF-4. (d) The percent relaxation recorded in aortic tissues treated with subfractions SF-1 and SF-2 of WF-4 fraction. Values are the mean ± SEM; *, **, and ***indicate significant difference compared with control-treated group at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. SEM: Standard error of the mean

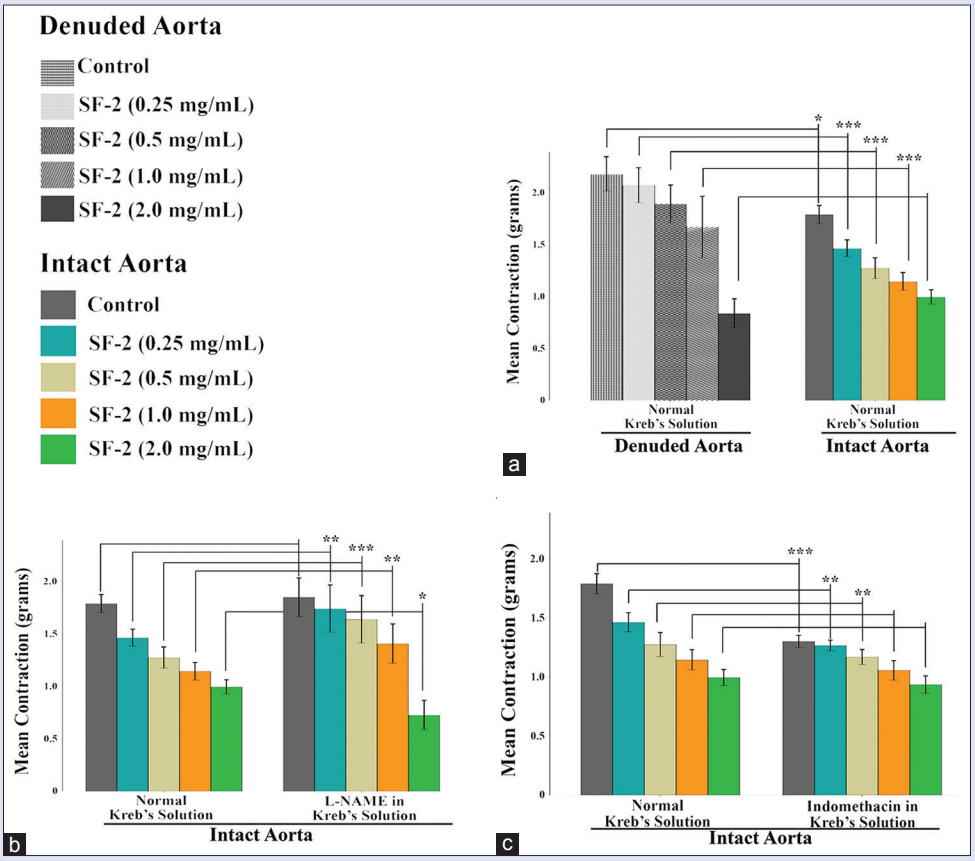

Figure 6: Statistical comparison between different concentrations of subfraction SF-2 on the contraction of aortic tissue in denuded and intact aorta (a), intact aorta with N-nitro-L-arginine methyl ester hydrochloride and without N-nitro-L-arginine methyl ester hydrochloride (normal Kreb's solution) (b), and intact aorta with and without indomethacin (c). Values are expressed as mean ± SEM; *, **, and ***representing the statistical difference within the groups with $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. SEM: Standard error of the mean

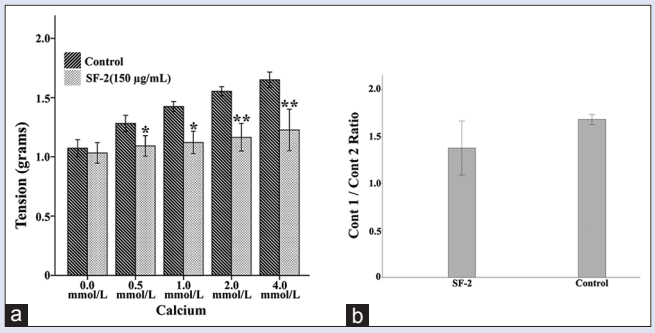

Figure 7: The effect of SF2 on calcium-induced contraction in isolated rat aortic rings prepared in calcium-free Kreb's solution. (a) The comparison of contraction recorded in rings incubated with norepinephrine hydrochloride in the presence of SF-2 (treatment group) and in the absence of SF-2 (control group). (b) The effect of ethylene glycol tetraacetic acid incubation on SF-2-induced relaxation. The ratio of contraction 1 (contraction in the aorta incubated with and without SF-2 in the presence of ethylene glycol tetraacetic acid) and contraction 2 (contraction in the aorta incubated with and without SF-2 in the absence of ethylene glycol tetraacetic acid) is presented in b. Values are expressed as mean ± SEM; **showing the difference of treatment group with control at the significance value $P < 0.01$. SEM: Standard error of the mean

on the 14th day of treatment, whereas the other two doses of PE showed a significant activity on day 7th ($P < 0.01$) and day 14th ($P < 0.01$). None of the three doses of ME lowered the BP on the 7th day, but the dose of 500 and 1000 mg/kg/day inhibited BP on the 14th day of treatment. CE was not found effective in all three doses, and the pressure recorded in CE-treated rats was not different from that of the control group rats. The results depicted that the antihypertensive effect of PM fruits could be attributed to its constituents with two different extremes in polarity. The activity was significantly contributed by the highly nonpolar constituents that resided more in PE as well as the highly polar constituents that were more concentrated in WE; however, WE showed maximum effect out of the four crude extracts that confirmed a more pronounced involvement of highly polar constituents of PM fruit in its overall antihypertensive effect. The hypotensive effect of ME in SHRs is recently reported;[15] however, our findings suggest that the WE is more effective than ME when the powdered fruits are extracted sequentially.

Arterial stiffness is one of the important cardiovascular parameters that affect the BP directly.[32] An increase in the endothelial destruction as well as the elevation of blood cytokine level with increasing age leads to a declined blood NO level, degeneration of elastin fibers, increased synthesis of endothelin-1, and proliferation of vascular smooth muscles, collectively resulting into an increased arterial stiffness.[33] Since the arterial stiffness is directly proportional to the PWV, the possible way to evaluate the impact of drugs on the arterial stiffness is the measurement of PWV. As the administration of WE exhibited maximum inhibition of NIBP in SHRs, its effect on the MAP, HR, and PWV was further investigated in SHR. For that, WE was administered to SHRs for 3 weeks. The rats were anesthetized on the 21st day and the three said parameters were recorded

by thoracic and femoral cannulation. The administration of a single dose of WE up to 500 mg/kg/day for 3 weeks did not show significant change in MAP and HR. Hence, the PWV in rats treated with WE up to 500 mg/kg/day was not different from that of the control group rats. However, at the dose of 1000 mg/kg/day, the extract significantly reduced MAP and HR in SHRs. This decline in MAP resulted into a significant decline in PWV ($P < 0.01$) that could be attributed to an improved endothelium of the vasculature over a period. Many antihypertensive drugs exhibit such decline in PWV apart from their antihypertensive effect. Treatment with angiotensin-converting enzyme (ACE) inhibitors such as perindopril is previously reported to have more pronounced effect on PWV than the calcium channel blockers.[34] A reduction in BP in SHRs by WE exhibited even more pronounced effect on PWV than the reference drug perindopril ($P < 0.05$).

Normotensive studies

WE was administered intravenously to anesthetized WKY rats through jugular vein, and the MAP and HR of the rats were recorded through pressure transducers connected with carotid artery. WE at the dose of 3, 6, and 12 mg/kg did not inhibit MAP and HR up to a significant level. However, at the dose of 25 mg/kg, it exhibited a significant fall in both MAP and HR ($P < 0.05$). The effect became even more pronounced at the higher doses of 50 and 100 mg/kg ($P < 0.001$). To investigate the possible underlying mechanism of this inhibitory effect of WE on MAP and HR, the effect of various cardiovascular drugs on the two said parameters was studied in the absence and presence of WE.

To test the effect of WE on α-adrenoceptors and calcium channels, the anesthetized WKY rats were administered with graded doses of NE-HCl through jugular vein before and after the administration of a single dose of 50 mg/kg WE, 1 mg/kg phentolamine (an α-blocker), and 1 mg/kg verapamil (calcium channel blocker) in separate groups of rats. The increase in MAP and HR by NE-HCl after the administration of WE, phentolamine, and verapamil was compared with the NE-HCl-induced elevation in MAP and HR before the administration of these drugs. WE showed a significant blockage of NE-HCl-induced elevation of MAP and HR in the similar fashion as exhibited by phentolamine and verapamil. The effects of NE-HCl on the heart are mainly mediated by α_1-adrenoceptors, while its effects on α_1-adrenoceptors in the blood vessels may contribute to increase in the MAP. Thus, the fact that WE reduced the increase in BP by NE-HCl suggests that it may inhibit α_1 or β_1-adrenoceptors or both. In addition, WE may also act as a calcium channel blocker such as verapamil to reduce the responses to NE-HCl. This was demonstrated by both phentolamine, an α_1-adrenergic antagonist, and verapamil, a calcium channel blocker. Moreover, to test the possible inhibitory effect of WE on the β-adrenoceptors, the rats were administered with graded doses of isoprenaline before and after the administration of a single dose of 50 mg/kg WE and 2 mg/kg propranolol (β-blocker) in separate groups of rats. Isoprenaline is a nonselective β-agonist that exhibits a dual effect. It increases HR through activation of β_1-receptors; however, it reduces the BP through the activation of β_2 receptors present on smooth muscles. Propranolol is a competitive β-receptor antagonist with equal affinity for β_1- and β_2-receptors, which blocks the actions of isoprenaline. The administration of propranolol blocked the isoprenaline-induced lowering of MAP and enhancement of HR in WKY rats. However, WE in the highest dose inhibited the increase in HR but potentiated the lowering of MAP by isoprenaline.

Vasorelaxant studies

Among the four crude extracts, PE and WE showed the strongest vasorelaxant effect which indicated the involvement of more than one type of vasorelaxant constituents; however, WE was the most potent vasorelaxant extract. To investigate the vasorelaxant effect of PE and WE, further, the two extracts were subjected to fractionation. The activity of the two fractions, PF-1 and PF-2 of PE, was evaluated on isolated rat aorta which depicted that although PF-2 fraction inhibited the NE-HCl-induced contraction significantly, the vasorelaxant effect of crude PE (up to 40% relaxation) was better than its fraction PF-2 which inhibited the contraction by 14% only.[35,36] It is suggested that the activity in PE was due to the collective effect of many constituents that were together in crude extract; however, the activity was reduced upon fractionation due to the separation of its constituents. This idea of synergistic effect of herbal constituents is well accepted and previously reported to result into a reduced biological activity upon fractionation.[16,35,36]

Although subfraction SF-1 of WF4 showed significant vasorelaxant effect at higher concentration of 2 mg/mL ($P < 0.01$), the subfraction SF-2 exhibited much stronger activity ($P < 0.001$) and hence the underlying mechanism was evaluated further in two different sets of experiments in the intact and denuded rat aorta. Vasorelaxation can be induced by two different sets of pathways. One pathway involves the inhibition of calcium influx through calcium channels on smooth muscle cells or the mobilization of calcium from intracellular calcium stores, whereas the other pathway involves NO or PG release from endothelial cells. The aorta was denuded to evaluate the effect of SF-2 on the smooth muscle calcium channels, the voltage-gated calcium channels (VGCCs), and the receptor-operated calcium channels (ROCCs). ROCCs are activated by high concentration of NE-HCl, while VGCCs are activated by high potassium concentration. When either channel is activated, the influx of calcium triggers the release of calcium from intracellular stores of calcium in the sarcoplasmic reticulum. All these activities result in increased amount of calcium in the smooth muscles, which cause smooth muscle contraction.

Incubation with SF-2 inhibited the calcium-induced contraction in the isolated aorta, supporting the inhibitory effect of SF-2 on ROCC channels. SF-2 possibly inhibited the calcium influx through ROCC that halted the tissue contraction. However, SF-2 did not attenuate NE-HCl-induced contraction in calcium-free medium when incubated with EGTA, indicating that it did not affect the release of calcium from intracellular stores. Hence, SF-2 was found to exert its vasorelaxant effect by blocking calcium influx, without affecting calcium mobilization from sarcoplasmic reticulum.

NO is synthesized by endothelial NO synthase.[37] The release of NO from endothelium increases the production of 3',5'-cyclic guanosine monophosphate (cGMP) by binding to soluble guanylate synthase in the adjacent vascular smooth muscle cells. Formation of cGMP-dependent protein kinases (PKGs) and subsequent phosphorylation of numerous proteins constituting cascade of protein phosphorylation/dephosphorylation lead to dephosphorylation of myosin light chain kinase and then relaxation.[38] PGI_2 is an eicosanoid that is being produced in vascular endothelium.[39] PGI_2 binds to its cell surface receptor (Gs-coupled IP-R) causing conformational changes and exchange of guanosine diphosphate for guanosine triphosphate on G-protein alpha (α)-subunit. It causes the release of inactive-bound trimeric G-protein from receptor and its dissociation into active α-subunit and beta (β)/gamma (γ) dimer. The α-subunit stimulates effectors such as adenylyl cyclase, thus elevating cyclic adenosine monophosphate (cAMP) levels,[40] which inhibits intracellular calcium levels in the vascular smooth muscle cells. High levels of cAMP activate protein kinase A and continue the cascade by phosphorylating myosin light chain kinase and inhibiting myosin light chain kinase, which lead to vascular smooth muscle cell relaxation.[41] The results of

the present study showed a significantly stronger vasorelaxant effect of SF-2 in the aorta with intact endothelium when compared to the denuded aorta ($P < 0.05$), thus concluding a possible role of vascular endothelium in the vasorelaxant effect of SF-2. It was concluded that SF-2 may induces NO release from endothelium that contributes to the vasorelaxant effect of SF-2 at concentrations ranging from 0.25 to 1.0 mg/mL; however, at higher concentration, the effect of SF-2 may involve other pathways such as blocking of calcium influx as stated above, and hence, its effect becomes independent of the endothelium. The effect of SF-2 in the group preincubated with indomethacin was also significantly different from the control group ($P < 0.05$). Thus, the vasorelaxant effects of SF-2 at concentrations range from 0.25 to 1.0 mg/mL in experimental models involving incubation with indomethacin, and L-NAME suggests the involvement of endothelium by both NO as well as PG pathway [Figure S9], while at higher concentration, the effect of SF-2 may involve other pathways such as blocking of calcium influx as stated above, and hence, its effect becomes independent of the endothelium.

The flavonoids and xanthones considered as secondary metabolites of plants are considered useful for the treatment of various cancer and cardiovascular diseases as shown by few *in vitro* and *in vivo* investigations in the previous literature. Quantitative and qualitative analysis of SF-2 by HPLC showed the presence of kaempferol 3-O-β glucuronide, gallic acid, rutin, and mangiferin in SF-2. However, the chromatogram of SF-2 showed many unidentified peaks, which needs further spectroscopic analysis. Xanthones such as mangiferin, flavonoids such as rutin,[42,43] kaempferol, and some of its glycosides such as kaempferol 3-O-β-D-glucuronide may have some preventive and therapeutic properties[44] associated with cardiovascular diseases as inhibition of ACE, inducing vasodilator effects,[45] antiplatelet effects, and antithrombotic effects. As SF-2 exhibited more efficient vasorelaxant effect in the intact endothelium as compared to the denuded aorta, this finding could be attributed to its constituent kaempferol that is previously reported to augment endothelial integrity by enhancing the release of NO from the endothelial cells.[46] Kaempferol induced NO release from the endothelial cells of the intact aorta that contributed to its vasorelaxant effect in addition to the inhibition of ROCC. Kaempferol also contributed to the reduced arterial stiffness in SHRs after 21 days of treatment by improving the endothelial integrity.

CONCLUSIONS

The antihypertensive and vasorelaxant effects of the PM may involve several mechanisms such as antagonism of α_1-adrenoceptors, inhibition of extracellular calcium influx, and release of NO and prostaglandins. In this study, the polar fraction and subfraction of PM WE showed pronounced reduction in BP, HR, and arterial stiffness in SHRs as well as in normotensive rats. Further investigation revealed that the antihypertensive effect is due to inhibition of the calcium influx, as well as inhibition of calcium mobilization from intracellular stores. The qualitative GC-MS analysis of the active fraction revealed the presence of four major constituents, i.e., kaempferol 3-O-β glucuronide, mangiferin, gallic acid, and rutin. Altogether, the present study demonstrated promising potentials of PM fruit to reduce the PWV, BP, and HR that could be attributed to the inhibition of arterial tone and extracellular calcium influx.

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Conflicts of interest

There are no conflicts of interest.

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